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Tuber sinoaestivum sp. nov., an edible truffle from southwestern China

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Abstract — A new *Tuber* species, *T. sinoaestivum,* is described from southwestern China based on morphological and molecular analyses. The new species, which was found fruiting in association with *Pinus armandii,* has been previously misidentified as *T. aestivum* (=*T. uncinatum*). Although morphologically quite similar, *T. sinoaestivum* differs from the European species by more globose ascospores ornamented with a shallower reticulum. ITS sequence analyses show only 92% similarity between the two species, further supporting the taxonomic separation of *T. sinoaestivum* from *T. aestivum*.

Key words —black truffles, *Pezizales*, phylogeny, taxonomy

Introduction

Chinese black truffles have attracted much scientific and commercial interest since their export to Europe in the beginning of 1990s. Four black truffle taxa have previously been reported from China: *Tuber indicum* complex, *T. formosanum*, *T. pseudohimalayense*, and *T. aestivum* (Chen et al. 2005; Chen & Liu 2011; Hu 1992; Manjon et al. 2009; Song et al. 2005; Wang et al. 1998, 2006; Zhang et al. 2005). The high morphological similarity between Chinese and European black truffles has prompted intensive investigations on species recognition and phylogenies (Bonito et al. 2010; Douet et al. 2004; Jeandroz et al. 2008; Paolocci et al. 1997; Roux et al. 1999). Previous studies indicate that a number of major *Tuber* clades are distributed across both China and Europe, yet species within the two regions appear to be distinct from each other.

T. aestivum Vittad. (= *T. uncinatum* Chatin), a renowned culinary species in Europe, is widely distributed across Nordic and Mediterranean regions (Jeandroz et al. 2008). This species was reported as a new Chinese record

based on ascomata sold at mushroom markets in Huidong, Sichuan (Chen et al. 2005; Song et al. 2005). Both identifications relied on morphological comparison with European collections, and spore size and ornamentation differences were regarded as mere intraspecific variations. In their worldwide phylogeny of *Tuber*, Jeandroz et al. (2008) did not include Asian *T. aestivum* material, leaving open the true identity of the Chinese collections. In recent years, more *T. aestivum*-like ascomata have been found in southwestern China. Morphological and ITS-rDNA and β-tubulin sequence comparisons of these Chinese truffles with European collections indicate that the Chinese material differs from *T. aestivum* and should be described as a new species.

Materials & methods

Morphological examination

Macroscopic characters are based on fresh and dried specimens. Descriptions and spore parameters follow Yang & Zhang (2003). Slides were made with a razor blade from dried ascomata and mounted in 5% KOH. Micro-morphological features were examined under a Nikon E400 microscope (10 × 100). At least 100 spores were measured from each ascoma and included asci containing different numbers of spores. For scanning electron microscopy (SEM), ascospores were scraped from dried ascoma gleba and mounted in distilled water on a cover glass. After air drying the cover glasses were directly attached to a SEM stub with double-sided tape and then coated with gold-palladium. The treated materials were examined and photographed with an AMRAY 1000B SEM. All specimens cited are deposited in the Herbarium of Cryptogams, Kunming Institute of Botany, Chinese Academy of Science (KUN-HKAS).

Molecular methods

Six truffle collections from China and seventeen from Europe were selected for phylogenetic analysis (Table 1). Total DNA was extracted from gleba dried with silica-gel using a modified CTAB procedure of Doyle & Doyle (1987). The ITS5 (forward) and ITS4 (reverse) primers were used to amplify the internal transcribed spacer regions (ITS-rDNA) of the nuclear ribosomal DNA (White et al. 1990) and Bt2a (forward) and Btspect (reverse) primers were used for the β-tubulin gene (Glass et al. 1995; Paolocci et al. 2004). The amplifications used 2 µl DNA template solutions in final volume of 25 µl. The final solution contained 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 2 mM $MgCl_2$, 200 µM of each dNTP, 0.2 µM of each primer and 3 U Taq Polymerase (Takara Biotechnology, Dalian Co. Ltd., China). The cycling parameters were an initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 52 °C (for ITS and 49 °C for β-tubulin) for 90 s and extension at 72 °C for 2 min, then a final extension at 72 °C for 10 min. PCR products were visualized through gel electrophoresis on a 1% agarose gel and purified with Watson's purification kit. Sequencing was performed with ABI Prism BigDye terminator cycle sequencing kit v3.1 on an ABI PRISM 3730 automatic sequencer. DNA sequences were edited and aligned with BioEdit Version 5.0.9 (Hall 1999) and Clustal X with manual adjustment where necessary. Maximum parsimony (MP) analysis was conducted with PAUP* 4.0b4a (Swofford, 2002) using heuristic

Table 1 *Tuber* sequences used in phylogeny.
(Bold font = sequences newly generated in this study.)

Voucher	Genbank label	Identification	GenBank		Locality
			# ITS	# β-tubulin	
——	*T. malenconii*	*T. malenconii*	FM205597	——	Spain
——	*T. malenconii*	*T. malenconii*	FM205596	——	Spain
E2	*T. aestivum*	*T. aestivum*	AF516781	AF516801	Italy
E61	*T. aestivum*	*T. aestivum*	AF516783	AF516812	Italy
E60	*T. aestivum*	*T. aestivum*	AF516789	AF516804	Italy
E5	*T. uncinatum*	*T. aestivum*	AF516791	AF516809	Italy
E24	*T. uncinatum*	*T. aestivum*	AF516792	AF516807	Italy
E17	*T. uncinatum*	*T. aestivum*	AY226042	AY206632	Italy
HKAS44347	*T. aestivum*	*T. sinoaestivum*	GU979038	GU979152	China
HKAS44322	*T. aestivum*	*T. sinoaestivum*	GQ217542	GU979150	China
HKAS70291	***T. aestivum***	***T. aestivum***	**JQ348410**	**JQ348402**	**Sweden**
HKAS70292	***T. aestivum***	***T. aestivum***	**JQ348411**	**JQ348401**	**Sweden**
HKAS70296	***T. aestivum***	***T. aestivum***	**JQ348412**	**JQ348404**	**France**
HKAS70297	***T. aestivum***	***T. aestivum***	**JQ348413**	**JQ348403**	**France**
HKAS59111	***T. sinoaestivum***	***T. sinoaestivum***	**JN896352**	**JQ348409**	**China**
HKAS59100	***T. sinoaestivum***	***T. sinoaestivum***	**JN896356**	**JQ348408**	**China**
HKAS59101	***T. sinoaestivum***	***T. sinoaestivum***	**JN896357**	**JQ348406**	**China**
HKAS59102	***T. sinoaestivum***	***T. sinoaestivum***	**JN896358**	**JQ348407**	**China**
HKAS70294	***T. mesentericum***	***T. mesentericum***	**JQ348414**	**JQ348405**	**France**
M13	*T. mesentericum*	*T. mesentericum*	AF516793	AY170363	——
M1	*T. mesentericum*	*T. mesentericum*	AF516794	AY170364	——
G02	*T. magnatum*	*T. magnatum*	AJ586251	AJ586430	Croatia
B6	*T. magnatum*	*T. magnatum*	AJ586271	AJ586428	Italy

search with multi-trees setting on and tree bisection reconnection (TBR) branch swapping with 1000 search replicates, each replicate with random sequence addition. No ambiguous characters were excluded from the analyses. Gaps were treated as missing data. Character states were treated as equally weighted and unordered. *Tuber magnatum* was selected as outgroup based on previous analyses, and *T. sinoaestivum, T. aestivum*, *T. mesentericum*, and *T. malenconii* comprised the ingroup taxa.

Results

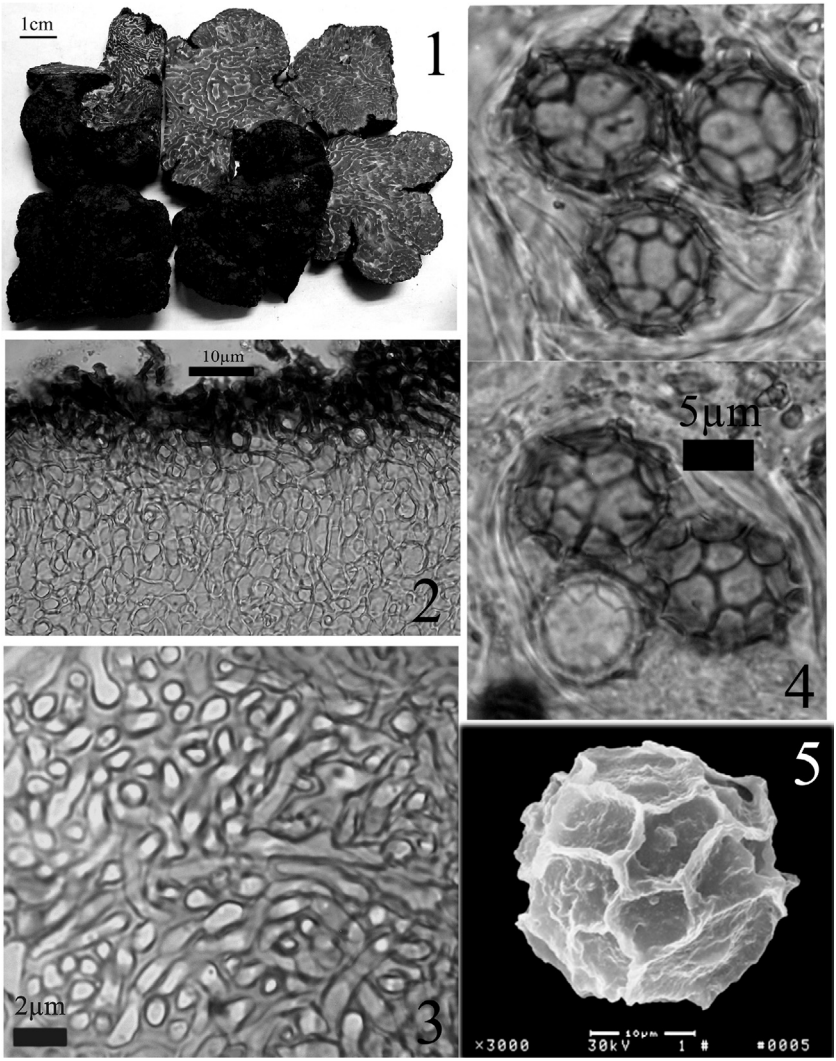
Taxonomy

Tuber sinoaestivum J.P. Zhang & P.G. Liu, **sp. nov**. Figs 1–5

MycoBank MB 563565

Differs from *Tuber aestivum* by its more globose spores with thicker walls and shallower ornamentation mesh.

Type: China, Sichuan Province, Huidong County, Gaji mountain, 26°29′N 102°31′E, alt. 2280 m, in *Pinus armandii* Franch. forest, 25 Nov. 2009, JP Zhang 153 (**holotype** KUN-HKAS 59105).



Figs 1–5. *Tuber sinoaestivum*. 1: Fresh ascomata; 2: Pseudoparenchymatous tissue of outer layer of peridium; 3: Intricately interwoven hyphae of inner layer of peridium; 4: Ascospores; 5: SEM of ascospore, showing the reticulate ornamentation.

Etymology: *sinoaestivum* (Lat.) referring to its Chinese origin and its relationship to European *T. aestivum*.

Ascomata subglobose, 1.5–3(–6) cm in diam., blackish to black, conspicuously covered with low, polygonal warts; warts 3–6-sided, 2–4 mm transverse, about 2 mm high, apex pointed. Peridium 100–620 μm thick, consisting of two

layers; the outer layer 50–300 μm thick, pseudoparenchymatous, composed of subglobose or subangular cells; cells dark to yellowish brown, 7–15 × 6–12 μm, thick-walled; the inner layer 50–350 μm thick, pale yellow or hyaline, interwoven hyphae 2–5 μm diam., forming an intricate texture. Gleba whitish at first when young, then becoming yellowish or brown gradually, marbled with numerous, narrow, white branching veins. Asci (51–)55–88(–124) × (33–)40–72(–75) μm (excluding stalk), subglobose, ellipsoid or variable in shape, sessile or with a short stalk, the stalk (6–)10–25(–50) × 5–15(–16) μm, 1–6(–7)-spored. Ascospores globose, subglobose, (17–)20–41(–47) × (15–)17–30(–35) μm [Q =1.04–1.50(–1.57), **Q** =1.15 ± 0.09], spore walls 2–4 μm thick, yellowish-brown at maturity, ornamented with a more or less irregular reticulum 2–5 μm deep; meshes variable, usually (7–)8–21(–22) × (4–)5–19(–20) μm, commonly 1–3 meshes in the transverse diam. and 2–4 meshes in the longitudinal direction. Odor unique with pleasant fragrance, taste a bit sweet for its mild aroma.

Habit and habitat: hypogenous, symbiotic with *P. armandii*, maturing from October to December or later.

Distribution: known only from southwestern China.

Additional specimens examined: ***Tuber sinoaestivum*** – **CHINA: Sichuan Province,** Huidong County, Daqiao mountain, 26°29′N 102°31′E, alt. 2250 m, 26 Nov. 2009, JP Zhang 158 (KUN-HKAS 59108); JP Zhang 156 (KUN-HKAS 59110); 19 Oct 2003, J Chen 129 (KUN-HKAS 44322). Gaji market, 23 Oct 2003, J Chen 141 (KUN-HKAS 44347). Gaji mountain, 26°29′N 102°31′E, alt. 2280 m, 25 Nov. 2009, JP Zhang 143 (KUN-HKAS 59095); JP Zhang 152 (KUN-HKAS 59104). Haiba mountain, 26°29′N 102°31′E, alt. 2300 m, 23 Nov. 2009, JP Zhang 140 (KUN-HKAS 59100); JP Zhang 141 (KUN-HKAS 59099); JP Zhang 144 (KUN-HKAS 59096); JP Zhang 149 (KUN-HKAS 59098). Yezu mountain, 26°29′N 102°31′E, alt. 2300 m, 27 Oct. 2009, JP Zhang 65 (KUN-HKAS 59101); JP Zhang 66 (KUN-HKAS 59102).

Tuber aestivum – **ENGLAND:** Hedben Bridge forest, 6 Aug. 2010, PG Liu (KUN-HKAS 70301, 70302). **FRANCE:** 2006, Y Wang (KUN-HKAS 70293, 70294, 70295); Lacoste, 46090 Le Montat, P Sourzat (KUN-HKAS 70296); La Devèze, 46090 Cours, P Sourzat (KUN-HKAS 70297). **ITALY:** Valle Fiorano (Modena), 10 Dec. 1984, A Zambonelli (KUN-HKAS 70298, 70299, 70300). **SWEDEN: Island of Gotland,** middle part of island, 8 Nov. 2003, C Wedén (KUN-HKAS 70290, 70291, 70292).

Phylogeny

Twenty-three samples were used for phylogenetic analysis. The homogeneity test for the conjoint analysis of ITS-rDNA and β-tubulin did not detect conflict between the ITS-rDNA and β-tubulin analyses, which allowed for a concatenated alignment and analysis. The alignment includes 1107 characters, of which 951 are constant, 149 are parsimony informative, and 7 are parsimony uninformative. Parsimony analysis generated one MP tree, with tree length = 360, CI = 0.928, RI = 0.968, RC = 0.898, and HI = 0.072.

In the concatenated analysis, the twenty-three samples grouped into three well-supported clades (Fig. 6): Clade I included all the Chinese materials

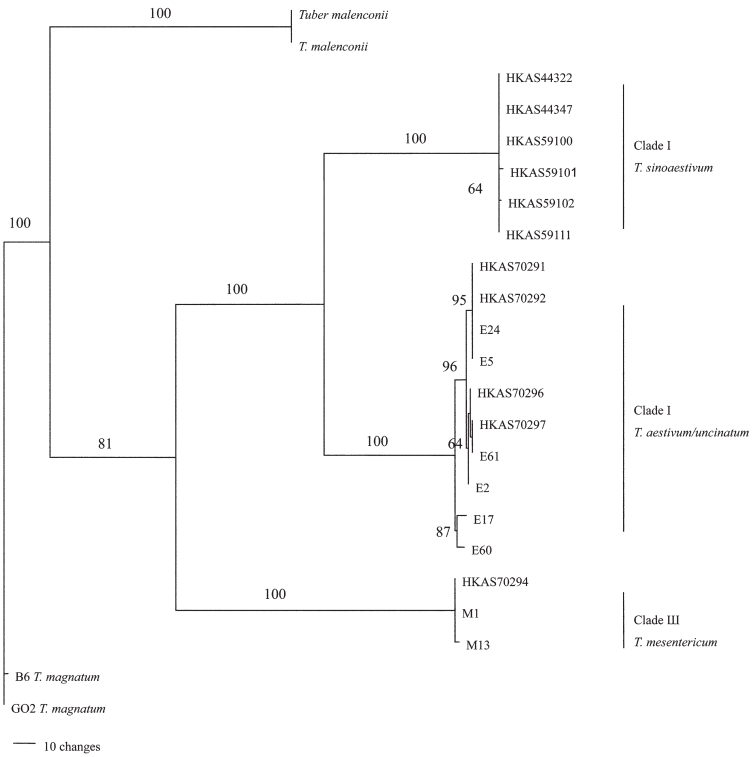


Fig. 6. Phylogenetic relationships of *Tuber sinoaestivum* and *T. aestivum* inferred from maximum parsimony (MP) analysis of ITS and β-tubulin sequences. MP Bootstrap values greater than 70% are indicated at nodes.

analyzed (*T. sinoaestivum*), Clade II included *T. aestivum/uncinatum* from Europe, and Clade III was represented by three *T. mesentericum* samples. Clade I formed a sister clade with Clade II. The same tree topology was obtained from the single gene ITS-rDNA and β-tubulin analyses but with different bootstrap values: the ITS-rDNA analysis shows 99% and 100% bootstrap support for the *T. aestivum/uncinatum* and *T. sinoaestivum* clades respectively, whereas the β-tubulin analysis indicates only 61% and 69% support. All analyses suggest a close relationship between *T. sinoaestivum* and *T. aestivum/uncinatum, T. mesentericum,* and *T. malenconii* but support a clear separation between the Chinese and the three European species.

Discussion

In the molecular phylogenetic tree, European *T. aestivum* collections (and others annotated as *T. uncinatum*) grouped together into clade II with 99% bootstrap support. The taxonomic relationship between *T. aestivum* and *T. uncinatum* has been subject to debate, with some regarding them as two species (Chevalier et al. 1994; Mello et al. 2002; Riousset et al. 2001) and others treating *T. uncinatum* as a morphological variation of *T. aestivum* (Sin et al. 1995; Trappe 1979), or perhaps two extremes of a morphological species-complex (Gandeboeuf et al. 1994; Pacioni et al. 1993; Urbaneli et al. 1998). Based on current research and our studies, we accept *T. aestivum* and *T. uncinatum* as a single widespread species with highly variable morphological and ecological characters. In addition, the phylogenetic trees suggest that "genetic variation" is not great. A previous multi-gene (ITS-rDNA, β-tubulin, EF1-α) analysis of sixty *T. aestivum* or *T. uncinatum* samples from Italy and other European countries has also supported *T. uncinatum* and *T. aestivum* as a single species (Paolocci 2004). A subsequent study by Wedén (2004) has drawn the same conclusion.

Tuber aestivum was initially reported from China as a new record because of its morphological resemblance to the European *T. aestivum* (Chen et al. 2005; Song et al. 2005). The Chinese collections that we studied are morphologically similar to the European *T. aestivum* in that both have black ascomata with conspicuous polygonal warts and reticulate spores. Such morphological similarity is mirrored in our ITS-rDNA and β-tubulin sequence analysis rooted by *T. magnatum* (Bonito et al. 2010; Jeandroz et al. 2008). However, after observing several European *T. aestivum* collections, we found that despite their almost identical macrocharacters, the two species are easily distinguished by spore shape. *Tuber sinoaestivum* spores are more globose (Fig. 7) as shown by the parameters in *T. sinoaestivum* [Q = (1.00–)1.04–1.40(–1.43), **Q** = 1.15 ± 0.11] in contrast to those in *T. aestivum* [Q = (1.03–)1.10–1.50(–1.65), **Q** =1.29 ± 0.13]. Furthermore, *T. sinoaestivum* spores have much thicker (2–4 μm) walls and shallower (<5 μm) meshes compared with the thinner walls (normally <2 μm thick) and higher (4–8 μm) meshes in *T. aestivum*. Chen et al. (2005) and Song et al. (2005) also noticed these differences but failed to use them for specific discrimination. European mycologists also noted the more globose spores in the Chinese *T. sinoaestivum,* but regarded it as a separate taxon (Zambonelli et al. 2012). Although *T. aestivum* is regarded as a widespread species with high morphological variation, the differences in *T. sinoaestivum* exceed the variation accepted for *T. aestivum*.

Until now, *T. sinoaestivum* has been found only from very limited localities in southwestern China. In the local Sichuan markets, it is often found mixed with collections representing the *T. indicum* complex. Zambonelli et al. (2012)

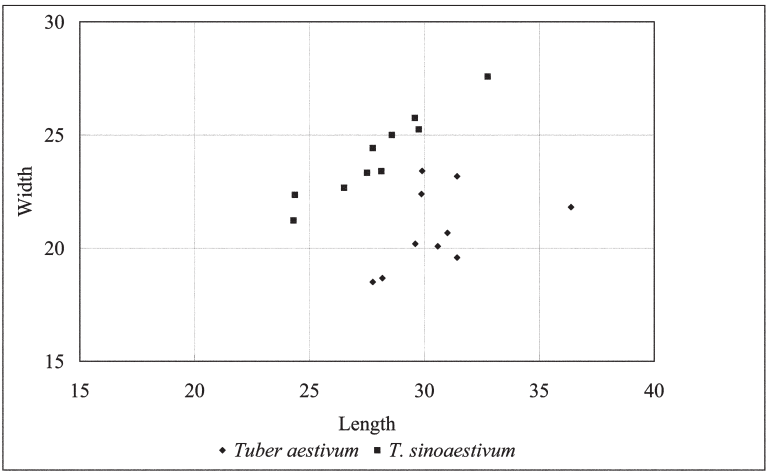


Fig. 7. Ascospore dimensions of *Tuber aestivum* and *T. sinoaestivum*; each point represents the mean values for 30 ascospores from a single ascocarp.

reported *T. sinoaestivum* (as "*T. aestivum* s.l.") as being sold in an Italian market. The very small number cited suggests that it could be mixed with fruiting bodies of *T. indicum* complex, a truffle intensively commercialized internationally. Potential introduction into *T. sinoaestivum* ecosystems cannot be excluded, as has occurred with *T. indicum* (Bonito et al. 2010; Murat et al. 2008), and precautions should be taken to avoid unwanted species invasions. Nevertheless, the culinary traits of *T. sinoaestivum* can still be commercially valuable. Accordingly, we suggest that when this truffle is traded, it should be classified and explicitly labeled *T. sinoaestivum* to emphasize its geographical origin and thus avoid confusion with *T. aestivum* and *T. indicum* complex. This will be significant for both the sustainable development and conservation of truffle biodiversity.

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